"FINDING THERMAL AND CONFORMATIONAL STABILITY OF IRON STORAGE NANOCAGE PROTEIN,FERRITIN BY CIRCULAR DICHROISM"

A thesissubmitted in partial fulfillment for the degree of

Integrated M.Sc in Chemistry

Submitted By:-

PRADEEP RATHORE

Roll no :- 410CY5097

National Institute of Technology, Rourkela, Odisha

Under the supervision of :-

Prof. Rabindra Kumar Behera

Department of Chemistry
National Institute of Technology, Rourkela-769008, Odisha, India
Telephone:-+91-9583484878

ACKNOWLEDGEMENT

This work is for the partial fulfillment of the requirements for Integrated Master of Science degree of the NIT University. Without the help, support and encouragement of some people this study would have not been completed.

My heartiest gratitude to my supervisor, ***Prof. Rabindra Kumar Behera****, Department of Chemistry, for providing me the opportunity to work in his laboratory. His wide knowledge, expertise and valuable suggestions provided an apt platform for learning and conducting research. I sincerely thank him for giving me so much of exposure, support and encouragement throughout my work.*

I would also like to thank all the members of Laboratory –Lab Technician ***Mr. Arup Dey****and the co-trainees-* ***Mr. Shantanu Mondal****for their constant support, help and encouragement.*

I would also like to thank the research scholars of Chemistry – ***Mr. Rahul Das,Miss Riyanka****and* ***Mr. Dinesh Nayak****for providing a friendly environment.I am grateful to my friends as well who had always given confidence and boosted my moral. I would thank all the people who directly or indirectly contributed to this effort whose names I might have unintensionally overlooked.*

My special thanks to ***Dr. Sunil Sarangi,*** *Director of NIT, Rourkela and* ***Mrs. AparnaMondal****, incharge of Project distribution, for getting me here to this reputed lab and showing me the way for valuable suggestion and moral support in my dissertation work.*

I have no words to express my thanks for the selfless support, encouragement, love and affectionof my ***parents****. I consider myself blessed to have such an adoring family and isindeedindebted to them for all that they have done for me. Last but not the least my acknowledgementwouldn'tbe complete without thanking* ***God****. I thank God for being my guiding light, and forgiving me the strength and encouragement to move ahead, inspite of all odds.*

(Pradeep Rathore)

CERTIFICATE

This is to inform that the thesis entitled “Finding thermal and conformational stability of iron storage nanocageprotein,ferritin by CD”which is being submitted by Mr. PradeepRathore roll no 410cy5097,for the award of the Degree Of Integrated Msc chemistry from National Institute of Technology, Rourkela,is a record for bonafied research work carried out by him under my supervision.The result embodied in this thesis are new and have not been submitted to any other institute or University for the award for any degree or diploma.

Prof. Rabindra Kumar Behera

CONTENT

INTRODUCTION

Ferritin is a globular intracellular protein complex that stores iron and releases it in a controlled fashion. The protein is produced by almost all living organisms, including algae, bacteria, higher plants, and animals. The molecular weight of Ferritin is 480 kDa consisting of 24 subunits that is present in every cell type.

Proteins carry out the most difficult tasks in living cells. They do so by interacting specifically with other molecules. This requires that the polypeptide chain fold to a unique, globular conformation that is only marginally more stable. The folded state is stabilized mainly by the tight packing of over 80% of the peptide groups and non-polar side chains.This is the structure that can be determined in crystals by X-ray crystallography.Proteins comprise an extremely heterogeneous class of biological macromolecules. They are often unstable when not in their native environments, which can vary considerably among cell compartments and extracellular fluids. If certain buffer conditions are not maintained, extracted proteins may not function properly or remain soluble. Proteins can lose activity as a result of proteolysis, aggregation and suboptimal buffer conditions.

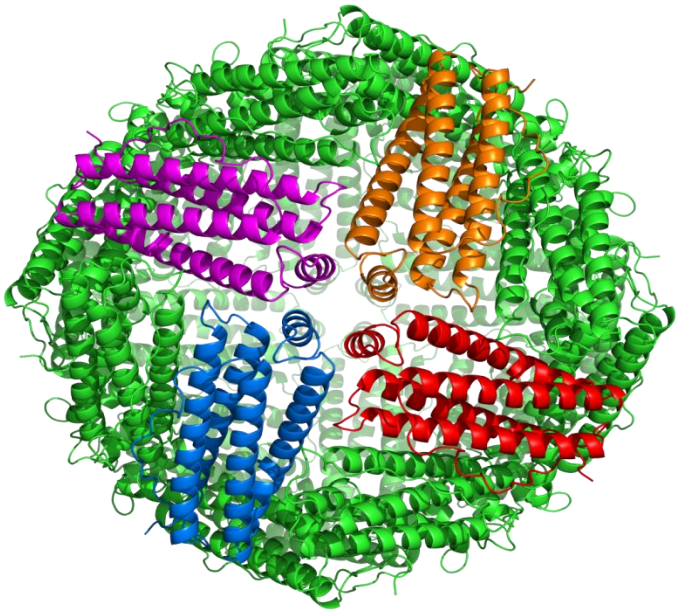


Fig. 1.Ferritin Structure showing 4 fold axis(PDB id – 1MFR). The diagram above shows the X-ray Crystal structure of Frog M Ferritin which contains 24 subunit.

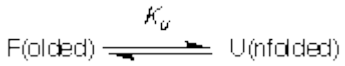
The stability of the protein is simply the difference in Gibbs free energy, ΔG, between the folded and the unfolded states. The only factors affecting stability are the relative free energies of the folded (G_f) and the unfolded (G_u) states. The larger and more positive ΔG_u, the more stable is the protein to denaturation.

$$\Delta G_u = G_u - G_f$$

The Gibbs free energy, G, is made up by enthalpy (H) and entropy (S), related by the equation:

$$G = H - TS$$

Protein stability primarily in terms of the **thermodynamic stability** of a protein that unfolds and refolds rapidly, reversibly, cooperatively, and with a simple, two-state mechanism:



Where K_u, is the equilibrium constant for unfolding.

The easiest proteins in which to study folding and stability are those that exhibit this sort of rapid reversibility.

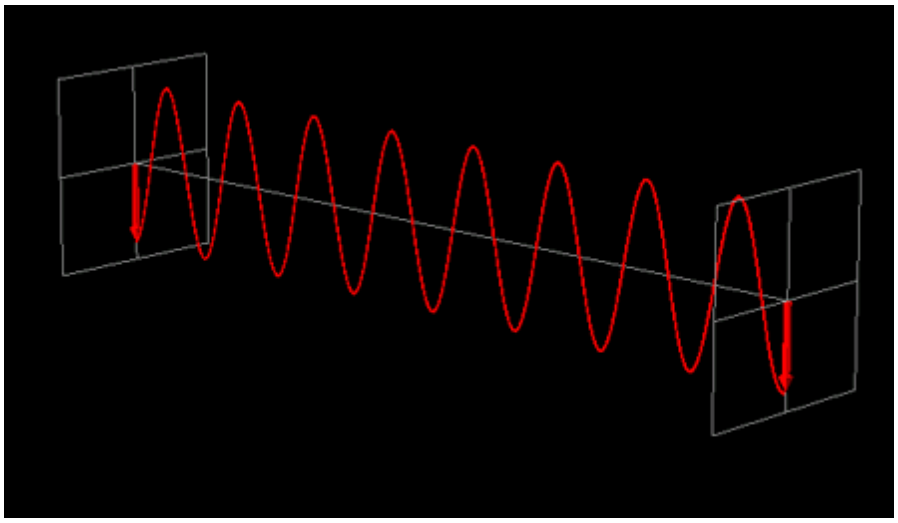
During stability measurements, a protein is gradually stabilized by changes in solution conditions, most commonly by elevation of temperature or addition of chemical denaturants. A major disadvantage of thermal unfolding is that it is often difficult to obtain true equilibrium data because proteins tend to aggregate at high temperatures, thus impeding thermodynamic analysis.

For many proteins, equilibrium unfolding induced by chemical denaturants such as Urea or Guanidine chloride (GdnHCl) is much better suited for quantification of stability because the salts bind to the amide backbone of the protein in such a way as to stabilize the unfolded protein. Moreover, chemical denaturation using urea or guanidine hydrochloride are often reversible, this means the system is at a true equilibrium, so a reliable Gibbs free energy, $\Delta G°$, of unfolding can be determined.

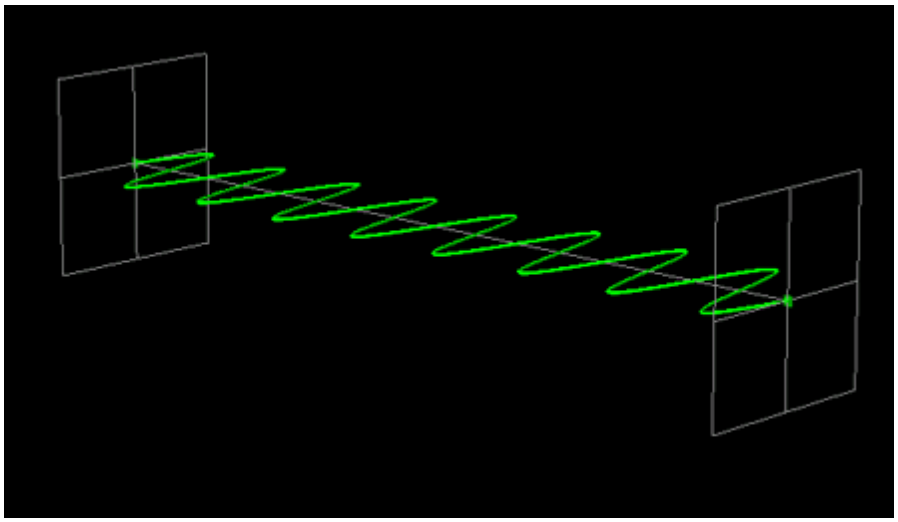
CD is a convenient and sensitive technique for monitoring the loss of protein structure. Combining this spectroscopic probe with chemical denaturation allows the ΔG° of unfolding to be determined with relative ease, and has therefore become a well-established technique for the study of protein stability.

Circular dichroism (CD) is the difference in the absorption of left-handed circularly polarised light (L-CPL) and right-handed circularly polarised light (R-CPL) and occurs when a molecule contains one or more chiral chromophores (light-absorbing groups). Circular dichroism = $\Delta A(\lambda) = A(\lambda)LCPL - A(\lambda)RCPL$, where λ is the wavelength.

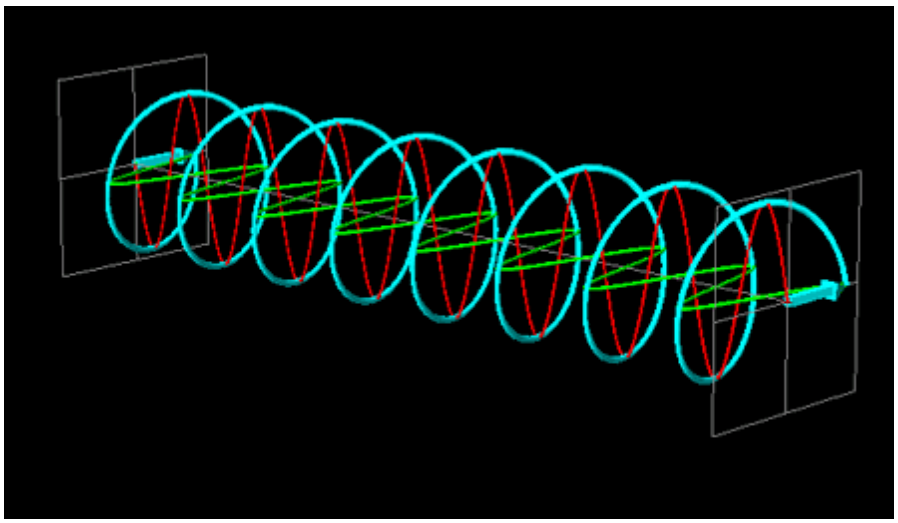
Circular dichroism (CD) spectroscopy is a spectroscopic technique where the CD of molecules is measured over a range of wavelengths. CD spectroscopy is used extensively to study chiral molecules of all types and sizes, but it is in the study of large biological molecules where it finds its most important applications. A primary use is in analysing the secondary structure or conformation of macromolecules, particularly proteins as secondary structure is sensitive to its environment, temperature or pH, circular dichroism can be used to observe how secondary structure changes with environmental conditions or on interaction with other molecules. Structural, kinetic and thermodynamic information about macromolecules can be derived from circular dichroism spectroscopy.



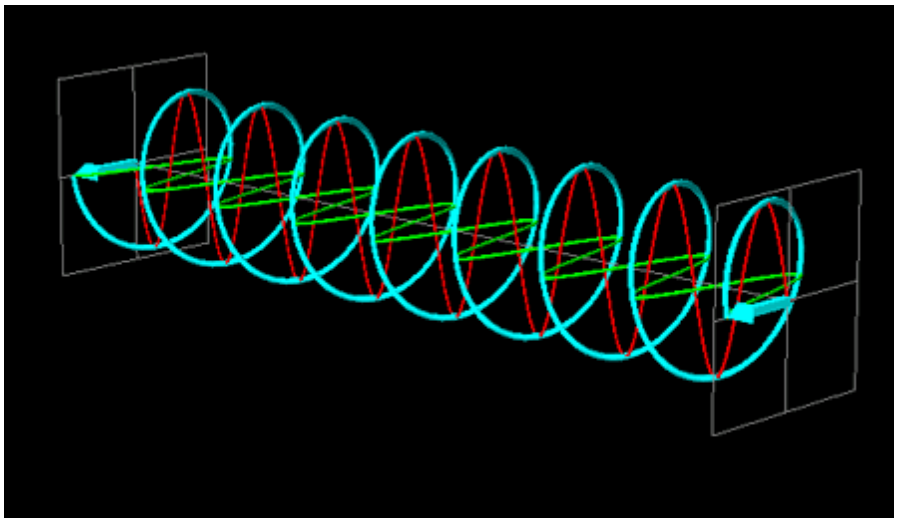
Vertically Polarised Light



Horizontally Polarised Light



Left Circularly Polarised (LCP) Light



Right Circularly Polarised (RCP) Light

Fig 2.**Circularly polarized light in CD.**The diagram above shows the vertically and horizontally polarized light and the left and right circularly polarized light. (adapted from Wikipedia)

The difference in absorbance of left-hand and right-hand circularly polarised light is the basis of circular dichroism. A molecule that absorbs LCP and RCP differently is *optically active*, or *chiral*.Circular dichroism (CD) spectroscopy measures differences in the absorption of left-handed polarized light versus right-handed polarized light which arise due to **structural asymmetr**y. CD is used for proteins because they are intrinsically asymmetric.The absence of regular structure (Unfolded protein) results in zero CD intensity, while an ordered structure (Folded protein) results in a spectrum which can contain both positive and negative signals.

The relationship between CD signal and the Beer-Lambert Law

$$\Delta A = A_{lcp} - A_{rcp} = (\varepsilon_{lcp}-\varepsilon_{rcp}) lc = \Delta\varepsilon$$

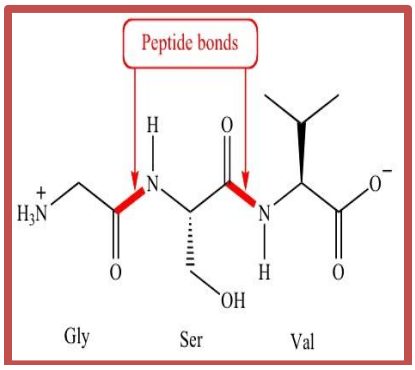
$$= CD(mdeg)/33000$$

Far UV CD (180 - 250 nm)

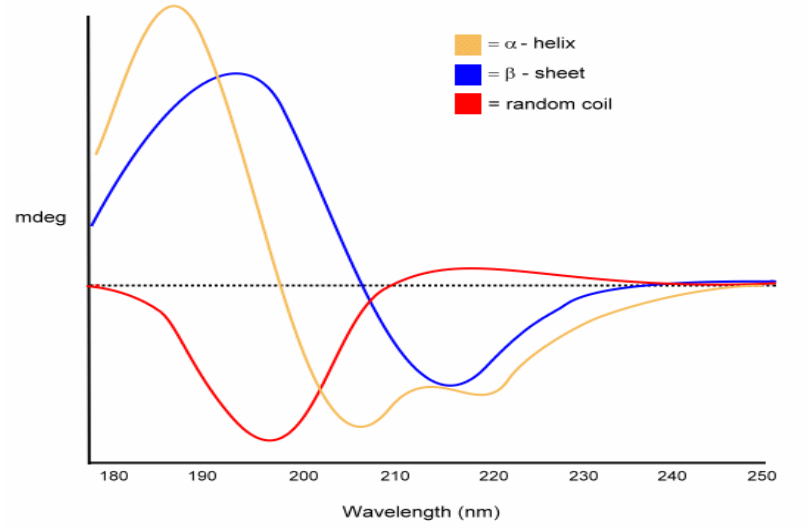
The amide group is the most abundant CD chromophore in proteins.

ππ* transition ~ 190 nm

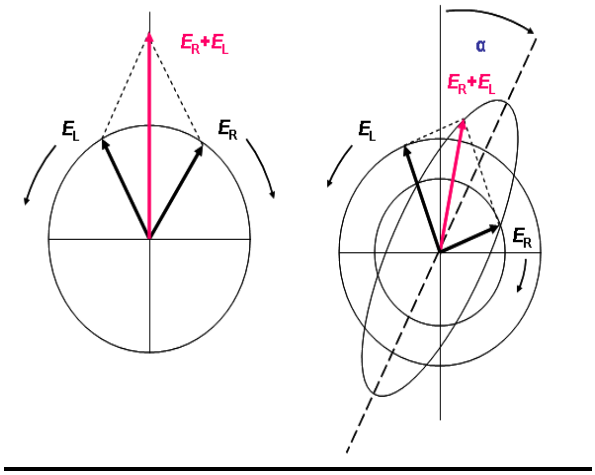
nπ* transition ~220 nm



In secondary structure conformations, the backbone amide bond chromophores are arranged in regular, organized, asymmetric patterns.



Linear (Plane) Polarized Light and CD



Linear polarized light can be viewed as a superposition of opposite circular polarized light of equal amplitude and in phase
Different absorption of the left- and right hand polarized component leads to ellipticity (CD)

MATERIALS AND METHOD

1. **Preparation of Buffers:-**

   - 25mM Tris (pH-7.4) containing 6.25mM NaCl –

     1M Tris was prepared by adding 121gm of Tris to 1L of distilled water and then it was diluted to 25mM. 3.6gm of NaCl was then added and mixed properly.

   - 8M Guanidine Hydrochloride (pH-7.4) –

     20gms of Guanidine Hydrochloride was dissolved in 25mM Tris and pH was adjusted to 7.4 by adding conc. HCl.

2. **Temperature Dependence CD unfolding study of Ferritin:-**

The Wild Type ferritin and M161H ferritin variant were selected for Temperature dependence experiment. The concentration of WT and M161H ferritin was measured by Bradford reagent.12μM Protein subunit concentration was taken and mixed with 25mM Tris 6.25mM NaCl (pH-7.4) buffer to a final volume of 1ml. This sample was then runned at JASCO CD instrument by setting different temperatures. The temperatures were 40°C, 45°C, 50°C, 55°C, 60°C, 65°C, 70°C, 75°C, 80°C, 85°C, 90°C and 95°C. The wavelength was set from 193-250$_{nm}$.

3. **Guanidine HCl induced unfolding study:-**

For unfolding study same Wild Type and M161H ferritin variant were taken. 8M Guanidine HCl was prepared in 25mM Tris(pH-7.4) containing 6.25mM NaCl buffer. 10μM Protein subunit concentration was taken and mixed with 25mM Tris 6.25mM NaCl (pH-7.4) buffer and GdnHCl to make a final volume of 1ml. The sample were prepared in different concentrations of GdnHCl and incubated for overnight at room temperature. The different concentrations were 1M, 2M, 3M, 3.5M, 4M, 4.5M, 5M, 5.5M, 6M, 6.5M, 7M, 7.5M and 8M. The wavelength was set at 222$_{nm}$. These samples were then runned at JASCO CD instrument.

RESULT AND DISCUSSION

1.Temperature Dependence CD Unfolding study of Ferritin:-

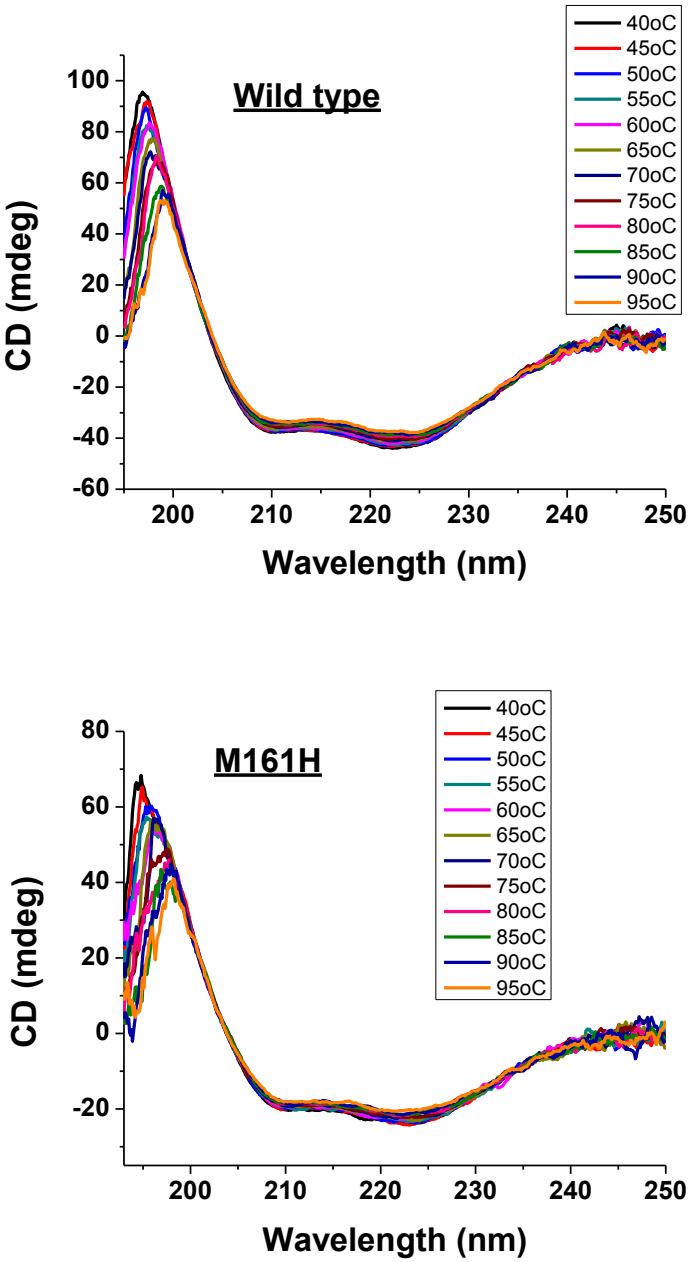


Fig 3. CD graph plotted in Origin, showing α-helical secondary structure in both WT and M161H ferritin protein.*** CD signal don't change much with increasing temperature**.

Structurally there was no change in WT and M161H ferritin variant at 95°C. These conclude that both WT and M161H act similarly and show no effect at high temperature. This experiment tells us that Ferritin protein is highly stable.

1. **GuanidiumHClinduced unfolding study:-**

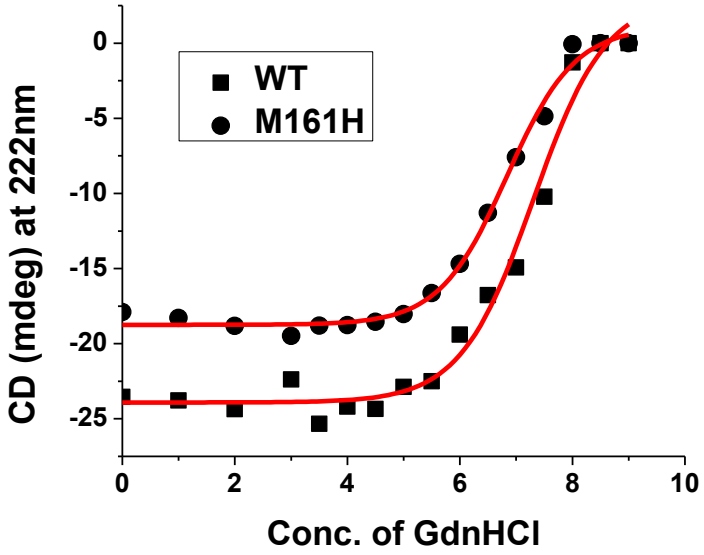


Fig 4.Graph plotted in origin showing guanidinium HCL induced unfolding study of both WT and M161H protein .

Wild Type protein, Conc. of GdnHCl required to unfold 50% is 7.3 M.

M169H protein, Conc. of GdnHCl required to unfold 50% is 6.8M.

***Histidine destabilizes ferritin structure.**

CONCLUSION

Ferritin is highly stable even at 95°C. Wild Type ferritin is conformationally stable compared to M161H mutant reflects hydrophobic interaction stabilizes ferritin nanocage.

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